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Full Length Research Paper

ELISA, RT-PCR, semi-quantitative RT-PCR and sequencing methods for investigating an epidemic FMD virus serotype O outbreaks

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This study was conducted to investigate the seroprevalence of non-structure protein for foot and mouth disease virus (FMDV) and identify FMDV serotypes. A total of 3600 serum samples (1080 buffaloes and 2520 cattle) were collected randomly from different breeds, age, and sex at El Beheira, El Dakahlia, and El Giza. Nonstructure protein of FMDV was detected in 41 of 1080 (3.8%) Buffalo and 185 of 2520 (7.3%) cattle. The case fatality was 14.6 and 17.8% in the vaccinated buffalo and cattle, respectively. The prevalence of nonstructural protein FMDV serotyping O was 8.3, 7.5 and 2.9% in El Beheira, El Dakahlia, and El Giza, respectively. Moreover, the case fatality was 8% (El Beheira), 26.3% (El Dakahlia), and 20% (El Giza). The circulation of FMDV was prevalent during the winter season of the year. The frequency of positive case was significantly different between species, sex, and age, while it was non-significant with different breeds. The mean values of antibodies of a non-structural protein of FMDV were the highest in male cattle at 5 months to 1 year of age. Additionally, seven epithelial tissues were collected from tongue; buccal mucosa and teat of recently sudden dead animals. The obtained sequences by reverse transcription polymerase chain reaction (RT-PCR) were registered in GenBank under accession numbers MF980930.1, MF991123.1, MF991124.1, and MF991125.1. Phylogenetic analysis revealed that the obtained sequences belonged to deposited FMDV type O (KP121442.1) with similarity ratios of 98, 100, 99, and 99%, respectively. Also, the deduced amino acids of the obtained sequences are related to capsid protein of *VP1* gene of FMDV.

Key words: Foot and mouth disease virus (FMDV), semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), VP1 gene, vaccinated animals, phylogenetic analysis.

INTRODUCTION

Foot and mouth disease (FMD) is the utmost spreadable disease of animals and has a great possibility for instigating serious economic damage in susceptible ruminant animals. The serotypes of FMD virus are O, A,

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C, SAT 1, SAT 2, SAT 3, and Asia 1. Infection with one type does not give immunity against another type of virus. FMD is difficult to differentiate clinically with other vesicular diseases. The diagnosis of FMD is an emergency case (Cox and Barnett, 2009; Alam et al., 2015; Saduakassovaa et al., 2018).

The ruminant animals are sensitive to FMD (FAO, 1984). The wild infected animal species transfer FMD Virus to the domesticated animal species. Also, camels have been reported to be infected with FMD Virus (Larska et al., 2009).

Foot and mouth disease virus belongs to family Picornaviridae and consists of seven different serotypes. Its RNA genome is about 8.5 kb that transcribes to 12 protein-coding genes and one viral genome protein (VPg) (Carroll et al., 1984; Forss et al., 1984; Li et al., 2007; Hwang et al., 2016; Abdulla et al., 2017).

Serotypes A, SAT2, and O of FMDV were detected in Egypt since 1950 and caused outbreaks during, 1953, 1958 and 1960 and till 2006 in livestock (OIE, 2000; Aidaros, 2002). FMD serotype A outbreaks were noted in cows and buffalos in 2006 (OIE, 2006; Knowles et al., 2007). Moreover, the outbreaks of serotype O were registered in 2006, 2007, and 2008 for Egyptian animals (El-Buhayrah and Alexandria governorates) even though the animal received for routine vaccination by a local bivalent vaccine which comprise both O1 and A/Egypt/2006 (FAO, 2008).

Egypt is an epidemic country of FMD that produces severe outbreaks almost every year and cause excessive economic damages (S1). The native routine vaccination of the animals is not professional for controlling the dispersal of the disease due to the lack of cross-protection between the different viral serotypes and subtypes (Mason et al., 2003) and the viral mutations are at the high rate, especially in *VP1* gene (Dopazo et al., 1988; Domingo et al., 1990; Samuel et al., 1999; Carrillo et al., 2005; Maryam et al., 2017; Islam et al., 2017).

The control of FMD should be quick, more perfect and constant via developed diagnostic tools to assess the circulating serotypes for limiting its diffusion into vast terrestrial areas (Belak, 2007; El-Khabaz and Al–Hosary, 2016). The application of reverse transcription quantitative polymerase chain reaction (RT-qPCR) is highly sensitive and is considered as a valuable tool for the detection of viruses to reducing the risk of cross-contamination (Mackay et al., 2002; Mackay, 2004; Howson et al., 2017; Haidar et al., 2018).

The present study aimed to investigate the seroprevalence of non-structural protein for foot and mouth disease virus (FMDV) and to identify its serotype in the collected samples. Also, it is performed to characterize and to determine circulating serotypes of FMDV during the risk time of year. On another hand, it was conducted to estimate the risk factor affecting the incidence of FMDV outbreaks.

Also, FMDV outbreaks cause the big losses for farmers and Egyptian national income, so this study is an attempt for early diagnosis of FMDV and advise to policymakers to develop an effective hexa vaccine against FMDV serotypes.

MATERIALS AND METHODS

Sampling

During the outbreaks of 2016 to 2017 in Egypt, a total of 3600 cattle and buffalo were investigated during the period between June 2016 and May 2017 in different farms located at El Beheira, El Dakahlia, and El Giza governorates (Figure 1). One hundred blood samples were collected from each governorate in a month. The susceptible animals composed of milking Friesian cows, buffalo and beef cow of different breeds, age, and sex.

In this study, a total of 3600 serum samples (1080 buffaloes and 2520 cattle) were collected from apparent and unapparent healthy animals of different breeds, age, and sex. Additionally, seven epithelial tissues were collected from the tongue; buccal mucosa and teat of recently sudden dead animals were post-mortems examined for the presence of tiger heart, swollen in a lymph node with hemorrhagic appearance and vasoconstriction of intestine leading to infection suspension with foot and mouth disease under national and international standard biosafety conditions and ethics.

All samples were collected from vaccinated animals with local Tri-Aphthovac® MEVAC vaccine which composed of inactivated FMDV serotypes (An Iran 05, O Pan Asia 2, and SAT-2) with oil adjuvant manufactured by ME-VAC.

The blood samples were left in tightly closed tubes overnight at 4°C and then centrifuged at 3000 rpm for 10 min to separate the sera and non-sera fractions. The clear serum was obtained by using sterile tips and placed in clean Eppendorf tubes, labeled, and stored at -70°C in the laboratory for the detection of antibodies to non-structure proteins of FMDV in animal serum samples. All positive non-structural protein samples were tested by indirect sandwich enzyme-linked immunosorbent assay (ELISA) for detection of FMDV antigen and serotypes.

Epithelial samples were collected in sterilized tubes that contained glycerol and phosphate-buffered saline (PBS), pH 7.2 to 7.6, (penicillin [1000 International Units (IU)], neomycin sulfate [100 IU], polymyxin B sulfate [50 IU], and Mycostatin [100 IU]). Samples stored at -80°C were used for isolation of the virus and identification for detection and confirmation using RT-PCR and sequencing methods.

Diagnostic tests

Serological diagnosis

Detection of non-structural protein of FMDV: Detection of IgG antibodies in animal's serum samples was carried out by FMDV 3ABC-Trapping ELISA to measure the antibodies of non-structure proteins of FMDV to differentiate between the antibodies against infection and antibodies against vaccination (Brocchi et al., 2006) produced by IZSLER, Biotechnology laboratory, via A. Bianchi, 9-25124 Brescia (Italy). These serological diagnoses were performed at the Animal Health Research Institute, Agriculture Research Center, Doki, Giza, Egypt.

FMD antigen typing detection ELISA Kits: All positive non-structural protein of FMDV samples were tested by indirect sandwich ELISA test for detection of FMD viral antigen and serotypes (Ferris and Dawson, 1988). The kits were produced at IZSLER Biotech Laboratory, Pirbright Institute, UK. These

Figure 1. Samples site of buffalo and cows from El Beheira, El Dakahlia and El Giza governorates.

serological diagnoses were performed at the Animal Health Research Institute, Agriculture Research Center, Doki, Giza, Egypt.

Molecular detection of FMDV by RT-PCR: The viral RNA was extracted from all collected samples by QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the mini spin protocol according to the manufacturer's instructions and eluted in 50 µl of elution buffer. 1 µg of the obtained RNA was used as the template in a one-step RT-PCR (Ready-To-Go RT-PCR Beads; Amersham). The primers used were F:5′.CCTCCTTCAAYTTACGGTG.3′ (Parlak et al., 2002) and R: 5′.GACATGTCCTCCTGCATCTG.3′ (Bachanek-Bankowska et al., 2016). The thermal profile was used as follows: 42°C for 30 min; 94°C for 5 min; 35 cycles of 94°C for 60 s; 55°C for 60 s; and 72°C for 90 s; followed by a final extension at 72°C for 5 min. The amplified RT-PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with Ethidium bromide using GeneRuler 50 bp DNA ladder (Cat. #: SM0373), then visualized under UV Transilluminator (BioRAD). The obtained RT-PCR products were purified by Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The RT-PCR products were eluted in nuclease-free water. The purified RT-PCR products were sent to Macrogen Company (South Korea) for sequencing service.

Semi-quantitative expression of VP1 gene in infected cows: Quantification of band intensities (OD) was measured using Image J software and a ratio OD candidate gene compared to OD of the control sample was calculated for each sample. The probability associated with one-way ANOVA.

Bioinformatics analysis

The obtained partial sequence for *Vp1* gene of FMDV was about 283 bp as shown in Figure 2. NEBcutter V2.0 software was used to create restriction map and to identify GC and AT ratios of the obtained sequence (Vincze et al., 2003; http://nc2.neb.com/NEBcutter2/). Jalview software was used to show SNPs that resulted from the alignment of the obtained sequences and the nearest sequences in NCBI database (http://www.jalview.org/). The obtained sequences were registered at GenBank under accession numbers MF980930.1, MF991123.1, MF991124.1 and MF991125.1 of Foot-and-mouth disease virus-type O isolates HM1, HM2, HM3, and HM4, respectively (http://www.ncbi.nlm.nih.gov). Construction of the phylogenetic trees was done by using Clustal Omega and MEGA7 software. Moreover, the amino acid sequences of the current sequences were obtained by using ExPASy translate tool (http://web.expasy.org/translate/). Also, the restriction maps of the current sequences were obtained through NEBcutter V2.0 tool (http://nc2.neb.com/NEBcutter2/) to display the possible endonuclease sites within the obtained sequences. GC and AT contents ratios were calculated by NEBcutter V2.0 tool of the obtained sequences.

Statistical analysis

The statistical analysis of deltamethrin DATA was done by one-way ANOVA and Independent T-tests using SPSS software version 20. The results were considered significantly different at $P<0.05$.

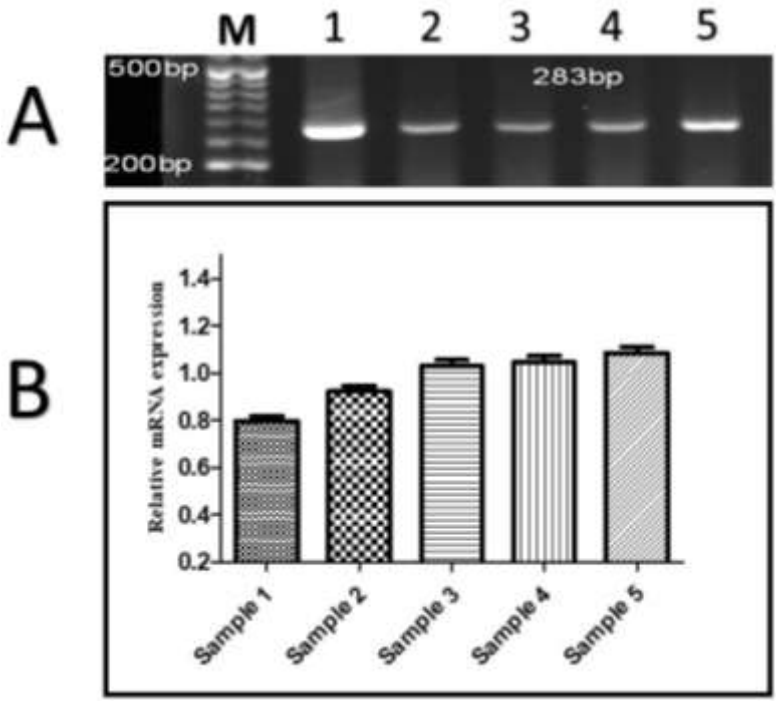

Figure 2. Differential expression of *VP1* gene levels in collected epithelial tissues of cattle for FMD. (A**)** Ethidium bromide stained agarose gel of purified clone of *VP1* gene with size of 283 bp (upper gel) compared to the control samples and M represented by 50 bp ladder. (B) Band intensity was quantified using Image J software and the ratio of OD *VP1* gene/OD of control sample was calculated after PCR.

Table 1. Incidence and case fatality (%) of FMDV in the vaccinated cattle and buffalo serum sample.

Species	No. of samples	No. of positives ELISA	Incidence (%)	No. of dead animals	Case fatality (%)
Buffalo	1080	41	3.8	6	14.6
Cattle	2520	185	7.3	33	17.8
Total	3600	226	6.2	39	17.2

Table 2. The prevalence of non-structural protein of FMDV in different governorates and serotype of FMDV.

Governorate	No. of samples	No. of positives	Prevalence (%)	No. of dead animals	Case fatality (%)	ELISA Serotype
El Behera	1200	100	8.3	8	8	O
El Dakahlyia	1200	91	7.5	24	26.3	O
El Giza	1200	35	2.9	7	20	O
Total	3600	226	6.2	39	17.2	O

RESULTS

The highest incidence and case fatality of FMDV were 7.3 and 17.8%, respectively in the vaccinated cattle serum samples (Table 1). The prevalence of non-structural protein serotyping O of FMDV was 8.3, 7.5 and 2.9% with case fatality 8, 26.3 and 20% in El Behera, El Dakahlyia, and El Giza governorates, respectively (Table 2). The incidence of FMDV was the most prevalent in the period between January and April (winter season) with

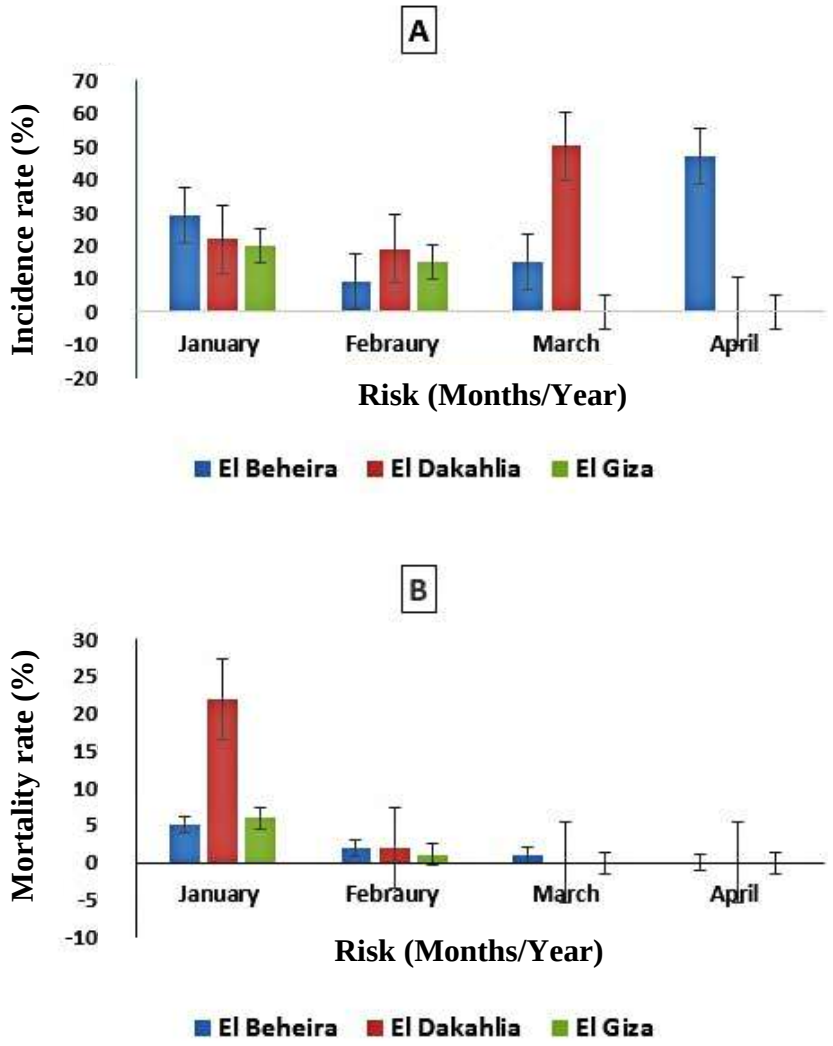

Figure 3. (A) The incidence rate of FMDV and (B) The mortality rate of FMDV at the risk time of year during the period of study from June 2016 to May 2017.

the highest mortality rate in January in all governorates during a period of the study (Figure 3). The frequency of positive case was significantly different between species, sex, and age; on the other hand, there was no significant difference with the breed. The mean values of antibodies of non-structural protein of FMDV were the highest in male cattle at 5 months to 1 year (Table 3). The obtained current sequences MF980930.1, MF991123.1, MF991124.1, and MF991125.1 were closed to foot-and-mouth disease virus-type o (KP121442.1) with high similarity ratios so they were located in the same clade (Figure 4). The single nucleotide polymorphism (SNP) showed little variation among the obtained current sequences (MF980930.1, MF991123.1, MF991124.1, and MF991125.1) and the nearest deposited sequence (KP121442.1) in DNA DATA base (Figure 5).

Moreover, the aligned protein sequences showed that the expected amino acids sequences, the current study, were related to polyprotein of foot-and-mouth disease virus-type O. The number of SNPs for the obtained amino acid sequences varied among the obtained sequences and the nearest ones registered in DNA database (Figure 6). This indicated that the obtained current sequences MF980930.1, MF991123.1, MF991124.1 and MF991125.1 related to deposited foot-and-mouth disease virus-type o (KP121442.1) in GenBank. Also, the collective phylogeny tree reported that the current sequences (MF980930.1, MF991123.1, MF991124.1 and MF991125.1) located in the same clade in the phylogeny tree. This revealed that the obtained sequences were more related to each other as shown in Figure 4. On the other hand, the obtained amino acids sequences converted from the current sequences showed that they belonged to VP1 gene which encoded to protein coat of foot-and-mouth disease virus-type o (Figure 6). The restriction map image showed many possible endonuclease sites of the

Table 3. Mean± SE of antibodies of non-structure protein of FMDV in the vaccinated cattle and buffalo serum sample.

Risk factor	Mean ± SE		
Species	**Buffalo** 1.30 ± 0.06[b]	**Cattle** 1.48 ± 0.04[a]	
Breed	**Balady** 1.42 ± 0.04[a]	**Frisian** 1.45 ± 0.05[a]	
Sex	**Male** 1.54 ± 0.04[a]	**Female** 1.10 ± 0.04[b]	
Age	**≤5 months** 1.54 ± 0.07[a]	**5 months to 1 year** 1.55 ± 0.05[a]	**1 to 3 years** 1.10 ± 0.04[b]

The means with different superscript in the same rows indicate significant difference; the significant difference is at the 0.05 level.

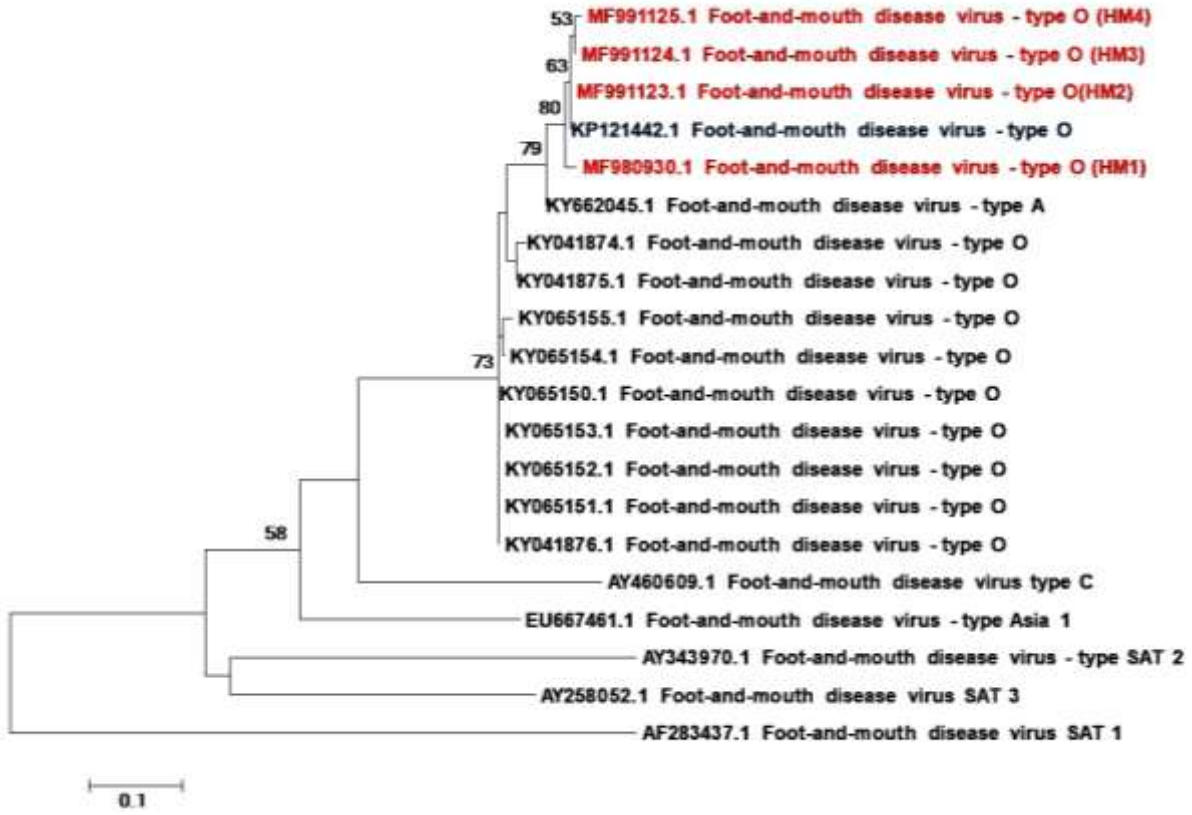

Figure 4. The phylogeny tree of the current sequences MF980930.1, MF991123.1, MF991124.1 and MF991125.1 showed that their location with foot-and-mouth disease virus-type O (KP121442.1) in the same clade with high similarity ratio. The Maximum Likelihood method was used to produce this tree by MEGA 7.0.21 software.

obtained sequences and GC and AT ratios. The expected endonuclease sites can be used as DNA markers for detecting foot and mouth disease virus in the future. The restriction maps revealed that endonuclease site (CTGAAG) of the Acu1 enzyme was common in the four obtained sequences. Furthermore, this common site sequence as single nucleotide polymorphism (SNP) with a PCR-RFLP application as specific SNP of foot and mouth disease virus type 0 can be exploited in the future survey studies.

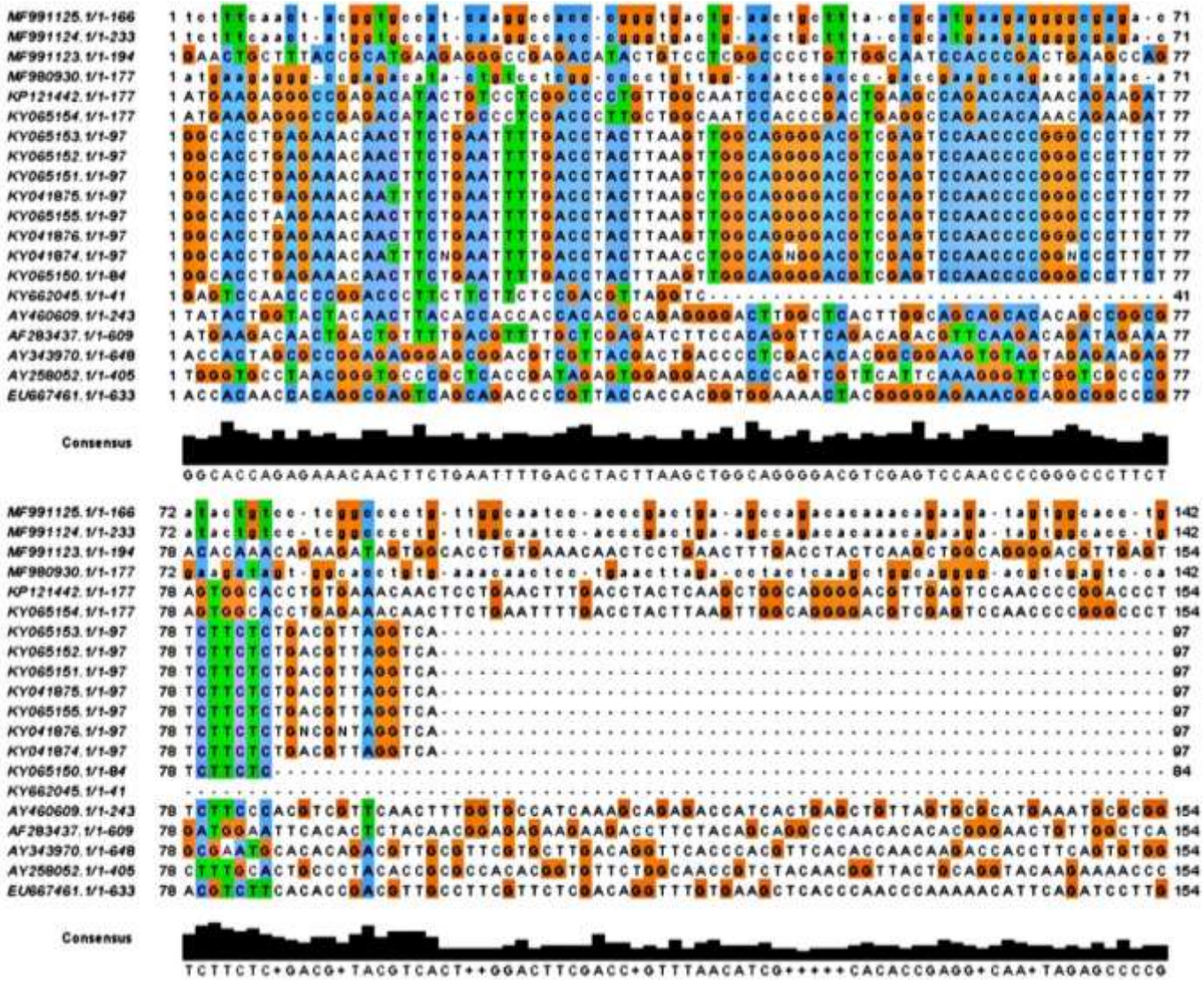

Figure 5. Single nucleotide polymorphism (SNP) image of MF980930.1, MF991123.1, MF991124.1 and MF991125.1 current sequences showing that there were many SNPs with the nearest sequence deposited in GenBank, Foot-and-mouth disease virus-type o (KP121442.1). This image produced usingJalview, version 2.10.1 software.

Semi-quantitative expression of *VP1* gene in infected cows showed little differences in the incidence of mRNA transcript levels among different dead cows; where the highest expression of *VP1* gene was shown in sample 5 as shown in Figure 2. The results of differential expression of *VP1* gene revealed that the FMDV was widespread in epithelial tissues of dead cows.

DISCUSSION

In Egypt, FMD has taken an enzootic form and many outbreaks had occurred since 1950 till now. FMDV type O was the most prevalent until serotype A appeared in 2006 (Moussa et al., 1984; Daoud et al., 1988; Farag et al., 2005; El-Khabaz and Al-Hosary, 2016); then during April and May 2012, six outbreaks of FMD type SAT 2 were reported in Egyptian governorates (Abd El-Moety et al., 2013; El-Khabaz and Al-Hosary, 2016). Up to date, several FMD outbreaks are still stroking the livestock in Egypt despite routine massive vaccination. The vaccination of livestock in Egypt with the triple vaccine appears not to be enough to control six FMDV serotypes. The vaccine should be included in all known serotypes to avoid any outbreak of FMDV. Then the big losses of farmers and national income can be reduced.

The serotype O of FMDV, in this study, was detected in El Behera, El Dakahlyia and El Giza governorates. IT was the most predominant in all regions from January to April 2017. FMDV mostly occured during winter as the

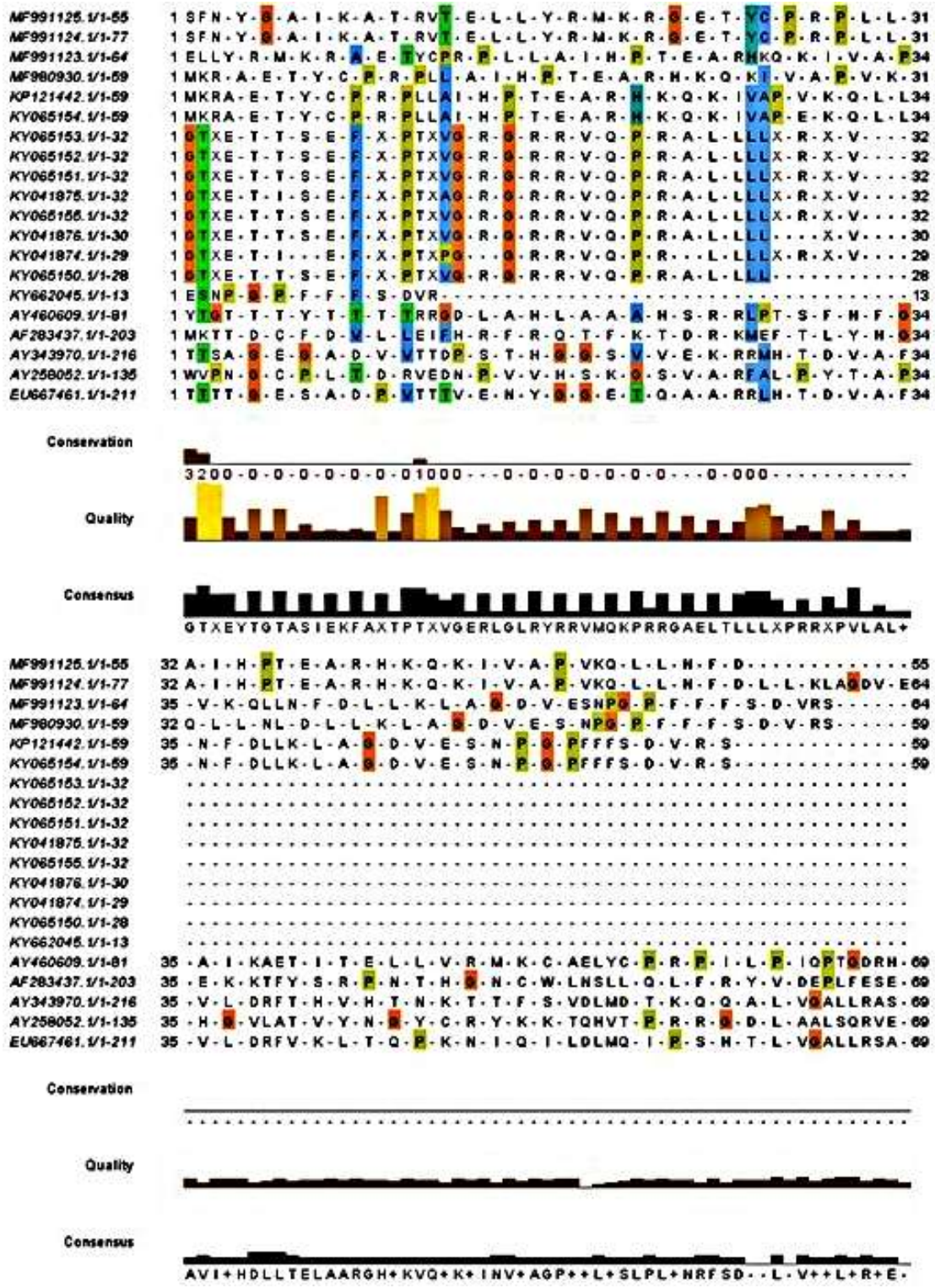

Figure 6. The consensus region of the obtained current amino acid sequences with the nearest ones registered in GenBank was obtained from Clustal Omega http://www.ebi.ac.uk/Tools/msa/clustalo/ and Jalview, version 2.10.1 software.

virus is sensitive to high temperature. Tomasula et al. (2007) and Sareyyüpoğlu and Burgu (2017) reported that at high temperature, there might be the destruction of virus receptors, which ultimately declined its infectivity.

The obtained results showed that the cattle were more sensitive than buffaloes to FMDV due to the fact that immunity of buffaloes is more than cattle. Also, the susceptibility of buffaloes to FMDV is less than cattle. This finding agrees with Ahmed et al. (2012) and Valdazo et al. (2012) who reported that the clinical picture of FMD in the affected animals was characterized by severe clinical signs in cattle, buffalos, small ruminants and young animals. These results agreed with Alexandrov et al. (2013) who reported that both mouth and foot lesions can occur in water buffalo, but the clinical signs are reported to be milder than in cattle and lesions may heal more rapidly, as its resistance is higher than that of cattle.

The obtained results clarified that 1 to 3 years old age is the least susceptibility to infection as may be attributed to the fact that they have a complete mature immune system and have previous exposure to FMD infection or vaccination. FMDV infected animals up to 5 months to 1 year of age. This may be attributed to colostral antibodies which protect animals to 3 to 6 months of age which in turn decrease the rate of FMDV infection among calves of less than 6 months of age (Alam et al., 2016). However, most deaths were among animals less than 1 year ago. This could be attributed to that naïve calves which develop more prominent clinical FMD signs and lesions than older animals including cardiac complications which are considered the main cause of high mortalities among younger animals (Geering and Lubroth, 2002). This also highlights the importance of proper vaccination of the dams to protect their young calves.

The prevalence of FMD disease was found significantly higher in male than female indicating that the male are more susceptible to FMDV than the female. The obtained results agreed with the previous findings of other authors (Alam et al., 2016; Mannan et al., 2009; Sarker et al., 2011; Rahman et al., 2015; Tomasula et al., 2007).

The obtained DNA data showed that the isolated FMDV serotype was type O. The phylogeny tree of the current sequences (MF980930.1, MF991123.1, MF991124.1, and MF991125.1) showed that they located with foot-and-mouth disease virus-type O (KP121442.1) in the same clade. Moreover, the differential expression of *VP1* gene confirmed that the cause of the death of cows was due to the spread of FMDV in the epithelial tissues.

Conclusion

Egypt is an epidemic country for FMD expressing many outbreaks of the disease almost every year producing excessive economic damages. The native routine vaccination of the animals is not professional for controlling the dispersal of the disease due to the lack of cross-protection between the different viral serotypes and subtypes and the high rate of viral mutations, especially in *VP1* gene; so the control of FMD requires quick, more perfect and constant well-developed diagnostic tools to assess the circulating serotypes for limiting its diffusion into vast terrestrial areas and will aid in the proper vaccine choice and consequently reduce disease damages. Moreover, ELISA, RT-PCR, semi-quantitative RT-PCR and sequencing methods are very informative for a screening of FMD virus and prediction of more effective vaccines.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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In vitro somatic embryogenesis and regeneration potential of two potato varieties in Uganda

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***In vitro* somatic embryogenesis and regeneration are important techniques for crop improvement and mass propagation. This study was conducted to establish and optimize a regeneration system for agronomically important potato cultivars ('Victoria' and 'Rutuku') of S*olanum tuberosum* L. in Uganda. Completely randomized design experiments were set up at Bioscience East and Central Africa (BecA) Tissue Culture Laboratory for this purpose. Callus induction and plant regeneration were initiated on internodes and leaf segments of the two potato cultivars *in vitro* on Murashige and Skoog (MS) medium supplemented with different phytohormones that included auxins such as α naphthalene acetic acid (NAA), cytokinins like benzyl amino purine (BAP), gibberellic acid (GA_3), 2,4-dichlorophenoxyacetic acid (2,4-D) and Zeatin at varying concentrations. Callus response depended on the genotype, the concentrations and composition of growth substances. Hormone combination MS + Sucrose 30% + NAA (2 mg/l) + 2,4-D (2 mg/l) + KN (2 mg/l) was found to have the highest callusing rate, that is, 95 and 53%, respectively for internodes and leaf explants. The calli formed from internodes and leaves were friable and soft. The callus colour in all cases ranged from light brown to light cream and in some cases to light green. Shoot bud initiation was observed in all regeneration culture media, with media combination MS + KN (2 mg/l) + Zeatin (2 mg/l) giving the highest shooting rate. The intervening callus phase led to less number of shoot buds for each callus leading to long incubation period. The study showed that it is possible to regenerate potato cultivars ('Victoria' and 'Rutuku') from cell suspension culture using Murashige and Skoog (MS) medium supplemented with different phytohormones. Combination of MS with NAA + 2,4-D + Kinetin is suitable for callus induction while MS + kinetin and zeatin was better for shoot induction on calli of potato cultivars 'Victoria' and 'Rutuku'.**

Key words: Regeneration, somatic embryogenesis, *Solanum tuberosum.*

INTRODUCTION

Irish potato (*Solanum tuberosum*) is an important staple food crop as well as a cash crop in the highland areas of Uganda where it is grown by over 300,000 smallholder households. Potatoes play a major role in national food and nutritional food security by providing a cheap but nutritionally rich staple food required in the fast growing population of Uganda, contributing protein, vitamins, zinc and iron to the diet (Abong et al., 2009). However, the productivity has stagnated between 5 - 7.5 t/ha at farmers level for many years while on-station yields go as high as

20 t/ha (FAOSTAT, 2014). This yield gap is attributed to pests and diseases (mainly late blight and bacterial wilt), low yielding varieties, poor disease management practices (Olanya et al., 2001), inadequate soil fertility management (Lemaga et al., 2001) and use of low quality seed potatoes (Byarugaba et al., 2017; Aheisibwe et al., 2015). With the rapid development of cell engineering, studies using suspension cells as starting plant material have gained momentum over the past several years. A fine cell suspension line is a good target tissue for gene transfer and somatic embryogenesis. Therefore, the establishment of *in vitro* reproducible regeneration protocols for cell suspension cultures in economically important potato cultivars provide an opportunity to make Biotechnology applications such as genetic engineering techniques more successful (Hussain et al., 2005).

Use of *in vitro* techniques such as somatic embryogenesis and regeneration combined with genetic engineering thus hold great prospect in addressing some of the production challenges for this crop especially diseases and seed quality (JayaSree et al., 2001). *In vitro* regeneration of plants have contributed to mass propagation of many plant species including potatoes and has emerged as an alternative for reducing cost of production (Vasil, 2012; Abbott and Belcher, 1986; Brar and Jain, 1998) as well as induction of fast crop improvements. Callus induction and subsequent plant regeneration using phytohormones accelerate the multiplication of young and strong plantlets.

In potatoes (*Solanum tuberosum L.*), different approaches have been tried to obtain efficient *in vitro* regeneration system from petioles with intact leaflets (Yee et al., 2001), leaves (Sarker and Mustafa, 2002; Andersson et al., 2003), leaf discs (Osusky et al., 2005), tuber discs (Vasquez and Clarence, 2002), and from stem (Chang et al., 2002) through callus induction. However, somaclonal variation in plants coupled with long regeneration period have been reported from potato plants derived from leaf discs (Fleming et al., 1992; Trujillo et al., 2001), stem segments (Cardi et al., 1993), anthers (Véronneau et al., 1992) and protoplasts (Sree-Ramulu et al., 1983; Coleman et al., 1990) and these have been reported to be genotype dependent. The development of somatic embryo has been done successfully using several explants tissues of potato such as tuber discs, nodes and leaf tissues on solid media (JayaSree et al., 2001). Since regeneration systems for most potato cultivars are genotype-specific, this limits their wide applicability to all genotypes (Ritchie and Hodges, 1993). Available evidence suggests that populations of regenerated plants may contain tremendous somaclonal variants which can be used to complement existing breeding programs. Studies have also shown that there is a strong link between the somatic embryos and the original explants tissue hence, production of somatic embryos from cell suspension cultures are more desirable. Due to differences in regeneration abilities of cultivars that are recalcitrant to various biotechnological advances (Sharma et al., 2008; Kumar and Kumar, 1996), identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration using *in vitro* cell culture, forms the key steps in potato genetic improvement program (Hoque et al., 2007). Therefore, genotype and nutrient media composition are the most important factors which affect callus induction and subsequent plant regeneration. Therefore, this study was conducted to establish and optimize a regeneration system for two agronomically important potato cultivars ('Victoria' and 'Rutuku').

MATERIALS AND METHODS

Potato explant materials and experimental design

Explants of two (2) potato cultivars of Victoria and Rutuku were obtained from Kachwekano Zonal Agricultural Research and Development Institute and taken to Bioscences for east and central Africa (BecA) Tissue Culture Laboratory. They were surface sterilized using sodium hypochorite (20%) and ethanol 70% for 5 min and rinsed 3 times with distilled water before cutting them into different segments of internodes, leaf disc and stems. The experiment was set up as a 3 factor factorial (media composition, Variety and explant type) and laid out in Completely Randomized Design (CRD) with 4 replications and 8 explants in each replicate.

Media used

MS basal medium (Table 1) was used and chemicals for preparation of the MS media were acquired from Duchefa. It contained the full macro and micro elements. The media was supplemented with vitamins (thiamine (Vit B1) 0.1 mg/l, Niacine (0.5 mg/l), Glycine (2.0 mg/l), pyridoxine (HCl) Vit B6 (0.5 mg/l) and Sucrose (25%), obtained from Sigma Aldrich inc. Plant growth regulators (phytohormones) were also obtained from Sigma Aldrich Inc and included mainly gibberellic acid (GA3), α-naphthalene acid (NAA), N-6-benzylaminopurine(BAP), indoleacetic acid (IAA), kinetin, 2,4-dichlorophenoxyacetic acid (2,4-D) and zeatin riboside.

For callus induction, leaf and internodal segments were put on callus induction media and maintained in the growth room at 16/8 light dark photoperiod at 18°C. The experiment was carried out with 4 replications with 5-8 explants in each replicate. The cultures were observed periodically for the development of the callus. Well-developed calli were excised and put on regeneration media containing different concentration levels of phytohormones (Table 1) for shooting and rooting and incubated at 25±2°C with a 16-h photoperiod.

For production of cell suspension, calli were put into liquid callus

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Table 1. Composition of the media used for callus induction and regeneration.

Media	Hormone combination
Callus induction	
M1	MS+ 2-4,D (2 mg/l) + NAA (2 mg/l) + KN (2 mg/l)
M2	MS+ 2-4,D (3 mg/l) + NAA (3 mg/l) + KN (3 mg/l)
M3	MS+ NAA (3 mg/l)
M4	MS+ 2-4,D (3 mg/l)
M5	MS (control)
Regeneration media	
M6	MS + BAP (1 mg/l) + IAA (1 mg/l)
M7	MS + BAP (2 mg/l) + IAA (2 mg/l)
M8	MS + NAA (1 mg) + BAP (1 mg/l) + KN (1 mg/l)
M9	MS + NAA (2 mg) + BAP (2 mg/l) + KN (2 mg/l)
M10	MS + BAP (1 mg/l) + NAA (1 mg/l)
M11	MS + BAP (2 mg/l) + NAA (2 mg/l)
M12	MS + KN (2 mg/l) +Zeatin (2 mg/l)
M13	MS (control)

production media with constant shaking at 150 rpm at 25°C. After two weeks of culture, the aggregated calli cells were transferred into 50 – 100 ml of fresh liquid MS media supplemented with NAA (2 mg/l) + 2-4,D (2 mg) and kinetin (2 mg/l). The cultures were maintained in complete darkness for a period of one month with sub-culturing every two weeks on fresh media. After removing the liquid, the aggregated cells in the flasks were removed and transferred to solid plant regeneration media supplemented with phytohormones as indicated in Table 1, followed by incubation in the darkness for 3 weeks at 18°C.

Data collection

Data were collected on callus induction frequency, days to callus initiation, callus texture, color and percentage of callus formation. Data were also recorded on the following parameters, days to shoot regeneration, number of shoots per explant, days to root induction, number of roots per explant.

Data analysis

Three factor factorial analysis of variance were performed using Genstat statistical software and mean separated by LSD at 5% level of significance.

RESULTS AND DISCUSSION

Effects of growth regulators on callus formation of two potato varieties

Regeneration was done using internodes and leaf segments as explants from two different potato cultivars namely: 'Victoria' and 'Rutuku'. The different phyto-hormones used in the preparation of media for regeneration were 2,4-D and naphthalene acetic acid (NAA) for callus induction while benzyl amino purine (BAP), zeatin and kinetin were used for shoot induction. The response to callus induction of internodes, and leaf segments of Victoria and Rutuku varied with genotype, explants type and the media composition. Initiation of callus was seen from internodal stem segments and leaf segments of Rutuku and Victoria in the media between 17 - 20 days after inoculation. All the explants formed callus (100%) but varied in the degree of callus development. A combination of MS+ 2-4,D (3 mg/l) + NAA (3 mg/l) + KN (3 mg/l) were found to be most effective auxin concentration level for callus induction in all the explant types for Victoria and Rutuku with greater than 60% of the explant forming friable callus within 40 days from the time of initiation (Table 2). These findings are in agreement with work done by Elaleem et al. (2009); Shirin et al. (2007); Castillo et al. (1998) who reported that 2,4-D and NAA can be used to enhance callus induction and maintenance at 3 mg/l as a growth regulator in MS media. It was also observed that media combination without Zeatin turned brown after prolonged incubation period. The media combination with Zeatin, turned green but took longer time (> 60 days) to initiate shoots from the callus.

For Rutuku, the best callusing rate was obtained from internodes (85 - 100%) compared to leaf segments (9 - 73.3%) while for Victoria internode callus rate ranged from 13 - 30% and for leaves 3 - 55%. In terms of texture, all the calli formed from leaf and internodes were friable and soft with a light to cream colour (Table 2).

Comparing the two types of explants, internodes of Rutuku gave a very good response to callus formation with callusing rate of 100% in M2 compared to other media compositions (M3, M4 and M5) (Table 2). Victoria also formed callus but the degree of callus formation was poor compared to that of Rutuku for both explants and in all media compositions (M1 - M5). The callus colour was gradually modified to light brown as it stayed on the media while on transfer to shooting media the colour (50%) of the callus changed to light green or light brown (Figure 1).

The result of the study shows that hormone combination of auxins [2, 4-D (2 mg/l) + NAA (2 mg/l) + KN (2 mg/l) is the most effective growth regulator for callus initiation in potato explants on Rutuku and Victoria (95/80%) compared to media combinations under M3, M4 and M5.

Effects of different phytohormones on shoot initiation of two potato varieties

Seven (7) weeks old calli of Rutuku and Victoria developed from the best callus media (Table 1) were transferred to regeneration media for development of the shoots. The two potato cultivars showed varying responses in shoot induction on different media composition. BAP + NAA at 3 and 5 mg/l caused

Table 2. Callus formation as influenced by different hormonal combinations.

Variety	Media ID	Media Composition	Days to callus initiation	Percent of callus formation (40 days after initiation)		Callus Texture	Callus colour
				Internode	Leaf		
Rutuku	M1	MS + 2-4,D (2 mg/l) + NAA(2 mg/l) + KN (2 mg/l)	19.5	95.0	53.8	Soft and friable	Light cream
	M2	MS + 2-4,D (3 mg/l)+NAA (3 mg/l)+ KN (3 mg/l)	20.0	100.0	60.0	Soft and friable	Light cream
	M3	MS + NAA (3 mg/l)	18.0	100.0	73.3	Soft and friable	Light cream
	M4	MS + 2-4,D (3 mg/l)	18.0	85.0	9.0	Soft and friable	Light cream
	M5	MS (control)	-	0.0	0.0	Soft and friable	Light cream
Rutuku (Mean)				**19.0**	**42.8**	Soft and friable	Light cream
Victoria	M1	MS + 2-4,D (2 mg/l) + NAA(2 mg/l) + KN (2 mg/l)	18.0	30.0	38.3	Soft and friable	Light cream
	M2	MS + 2-4,D (3 mg/l) + NAA (3 mg/l) + KN (3 mg/l)	17.0	5.0	55.0	Soft and friable	Light cream
	M3	MS + NAA (3 mg/l)	18.0	30.0	10.0	Soft and friable	Light cream
	M4	MS + 2-4,D (3 mg/l)	17.3	13.0	3.0	Soft and friable	Light cream
	M5	MS (control)	-	0.0	0.0	Soft and friable	Light cream
Victoria (Mean)				**17.4**	**19.2**	Soft and friable	Light cream
Grand Total				18.0	30.5	Soft and friable	Light cream
F.pr (5%)				0.024			
LSD				17.49			

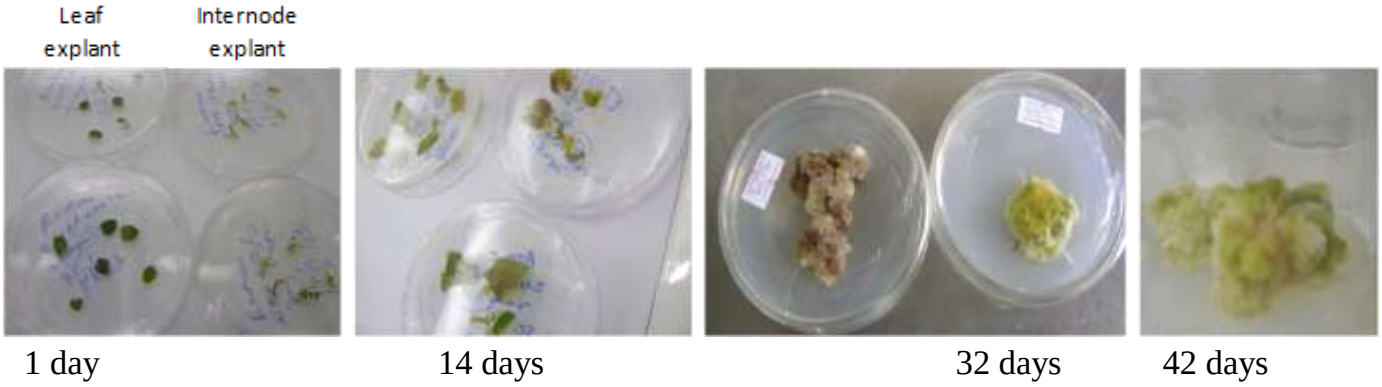

Figure 1. Stages of callus formation from internodes and leaf segments.

calli from both varieties to grow in size with some calli developing shoots (Figure 2) while in some cases, no regeneration was observed even after 50 days on culture as shown in Figure 1.

In all these cases, intervening callus phase resulted in initiation of very few numbers of shoots from the callus and where shoot induction occurred, there was a prolonged period of incubation.

Based on this study, it was found out that use of NAA in combination with BAP is good for maintenance of the callus and callus growth at 3 mg/l. The results of the study shows that the callus from Victoria grew much bigger in size compared to Rutuku (Figure 2) and this was attributed to difference in the genotype of the two varieties.

Application of MS + BAP (2 mg/l) + IAA (2 mg/l) at 2 mg/l and 1 mg/l each resulted in the calli developing roots instead of shoot for both Rutuku and Victoria. The calli became light brown in colour and the developed roots became hairy and elongated with size over 3 cm due to the action of these hormones. This implies that combination of Zeatin and BAP should be applied after

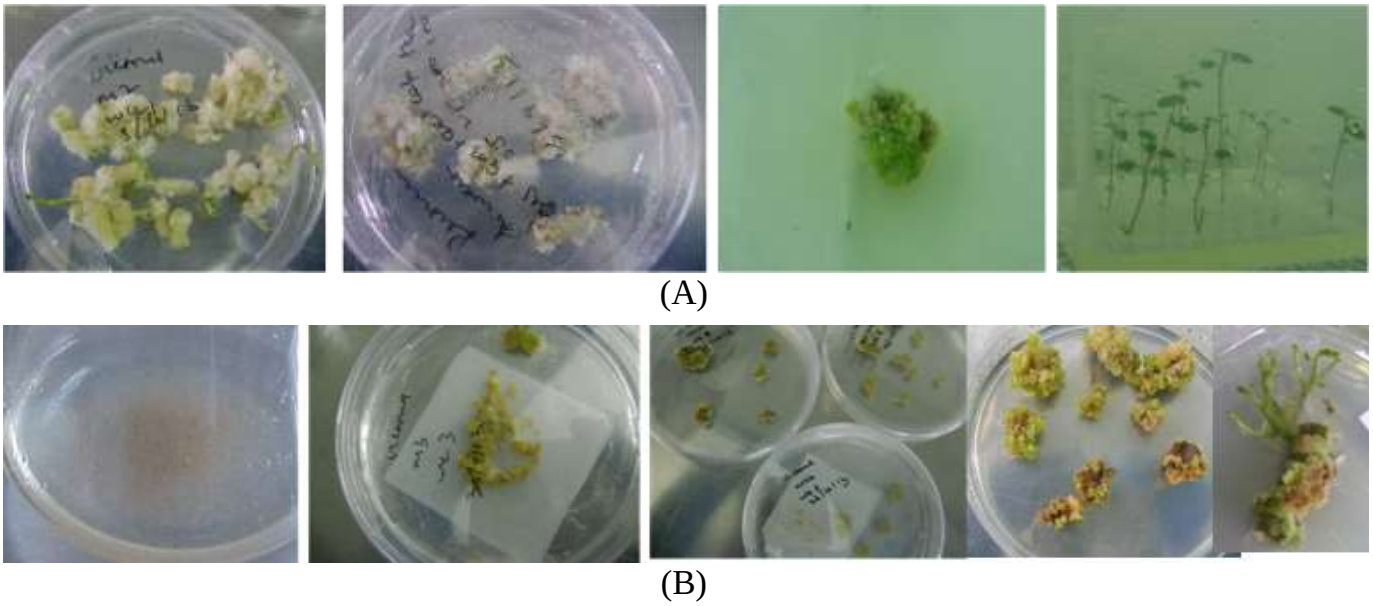

Figure 2. Development of somatic embryos from (A) callus and (B) cell suspension.

Table 3. Effect of growth regulators on shoot induction.

Media ID	Media composition	% Shoot regeneration from callus		
		Rutuku	Victoria	Grand Mean
M6	MS+BAP (1 mg/l) + IAA (1 mg/l)	18.3	6.3	13.0
M7	MS+BAP (2 mg/l) + IAA (2 mg/l)	16.7	25.0	20.2
M8	MS + NAA (1 mg) + BAP (1 mg/l) + KN (1 mg/l)	27.8		27.8
M9	MS + NAA (2 mg) + BAP (2 mg/l) + KN (2 mg/l)	16.7	0.0	6.7
M1O	MS + BAP (1 mg/l) + NAA (1 mg/l)	40.0	12.5	32.1
M11	MS + BAP (2 mg/l) + NAA (2 mg/l)	5.0	12.5	7.1
M12	MS + KN (2 mg/l) + Zeatin (2 mg/l)	26.7	42.7	34.7
M13	MS (control)	6.7	0.0	3.3
Grand Mean		20.0	13.9	17.6
F.pr		0.037		<0.001
LSD		18.41		

the shoots have developed and not before shoot development as its action suppresses shoot development when applied at callus stage. In all these cases, intervening callus phase resulted in initiation of very few number of shoots from the callus. Where shoot induction occurred from callus, there was a prolonged period of incubation and this could result in undesirable somaclonal variations (Table 3).

The best media combination was noted as M12 with 34% shoot regeneration. This media component contained Zeatin riboside that is good for controlling the development of highly organogenic micro calli and supports both organogenesis and somatic embryogeneis. This result is supported by the report of Beaujean et al. (1998) and Ghosh et al. (2015) that noted that Zeatin when applied at 0.8 mg/l stimulates shoot development. However, in this case, the optimum rate of application was observed at 2 mg/l in combination with Kinetin at 2 mg/l.

Conclusion

A reliable regeneration system has been established for two potato cultivars 'Victoria and 'Rutuku' This regeneration system can be applied in the genetic

improvement targeting the most important diseases mainly late blight and bacterial wilt that are heavily ravaging the potatoes in Uganda. This study also shows that other potato varieties can be regenerated by optimization of the media components especially phytohormone concentration to enable regeneration and transformation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Enzymatic activity and elicitor of phytoalexins of *Lippia sidoides* Cham. and endophytic fungi

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Secondary metabolites may play a number of roles, among them, is the activation of plant defense mechanisms, such as phytoalexins and the synthesis of antioxidative enzymes. This study aimed to test the eliciting activity of phytoalexins of *Lippia sidoides* Cham. and six endophytic fungi associated with it and to verify the synthesis of the enzymes, as a response to the antioxidative and antifungal system. The assays were performed using spectrophotometric methods. Of the concentrations of essential oil used, 7.5 mg mL^{-1} was 66.48% higher than the Acorda® positive control for the induction of glyceollin. The endophytic fungus, *Verticillium* sp. showed the best result for 3-deoxythianocyanidine production. Excellent results were demonstrated by the LS-14 fungus for superoxide dismutase enzyme activity (130 U g^{-1} E^{-1} min^{-1}); by *L. sidoides* for the activity of ascorbate peroxidase enzyme (3821 µmol ASA g^{-1} E^{-1} min^{-1}); by *L. sidoides* for the activity of catalase enzyme (2429 µmol H_2O_2 g^{-1} E^{-1} min^{-1}); by *Fusarium* sp. 2 for the activity of phenol peroxidase (87 µmol H_2O_2 g^{-1} E^{-1} min^{-1}) and by *Fusarium* sp. 1 for the activity of chitinase (0.214 U min^{-1}). Both the essential oil of *L. sidoides* and the extracts of the endophytic fungi had a positive response to the induction of phytoalexin and presence of enzymes related to antioxidative activity were verified and shown.

Key words: Catalase, superoxide dismutase, peroxidase phenols, ascorbate peroxidase, chitinase.

INTROUCTION

With increased productivity in agriculture and, consequently, increased use of agrochemicals, mainly related to the control of plant diseases, damage to the environment and living beings has appeared (Matiello and Bonaldo, 2013). However, the use of alternatives to conventional chemicals such as the use of plant extracts, fungi and essential oils for phytopathogenic control has been highlighted (Oliveira et al., 2016).

Compounds such as kauralexins and zealexins in *Zea mays* and oryzalexin E in *Oriza sativa* have the potential to control phytopathogens, both by their direct fungitoxic action and by the ability to induce the accumulation of

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phytoalexins by activating plant defense metabolism (Venturoso et al., 2011). They are an interesting strategy in commercial agriculture, to develop biotechnological applications in the protection of plants (Ribera and Zuñiga, 2012). The tools could be used in the production of plants that express a greater amount of phytoalexins induced by both, phytoalexin elicitors or by genetic transformation (González-Lamothe et al., 2009).

When plants come in contact with so-called elicitors, their defense system is activated, which induces resistance (Smith, 1996). The presence of these molecules, demonstrates antimicrobial activity with important actions in the protection of plants since they do not have an immune system like the vertebrates (Jenssen et al., 2006). They can also be called phytoalexins, which are synthesized in cytoplasmic inclusions near the site of attempted entry of the pathogen. In fungi, they disorganize the cellular content; the plasma membrane is ruptured and fungal enzymes are deactivated (Peiter-Beninca et al., 2008).

Phytoalexin glyceollin in soybean (*Glycine max* L. Merr.) protects this legume. It is often reported in induction studies, for the use of its cotyledons, a tool used for studies involving the eliciting action because they are easily measured (Schwan-Estrada et al., 2000). Also, the use of etiolated mesocotyls of sorghum (*Sorghum bicolor* L. Moench21), inducing the production of phytoalexin 3-deoxythyanocyanidine promotes the increase in plant tissue resistance (Matiello and Bonaldo, 2013). It is also used in bioassays to test the elicitor effect of a treatment.Under stress conditions, the plants activate antioxidative defense mechanisms, such as resistance, in order to neutralize the cytotoxicity provoked by reactive oxygen species (ROS). In this system, the enzymes catalase (CAT), ascorbate peroxidase (APX), phenol peroxidase (POX) and superoxide dismutase (SOD) (Miller et al., 2010) are evidenced. Besides phytoalexins, these enzymes can have effect on plants resistance to pathogens.

Peroxidases catalyze the oxidation and eventual polymerization of hydroxycinnamic alcohol in the presence of hydrogen peroxide, which can lead to lignin. In addition, they participate in suberization, formation and cross-linking of cell wall components, auxin catabolism, senescence, protection against the attack of pathogens, insects and abiotic stressors (War et al., 2012). SODs remove superoxide radicals by preventing them from crossing the lipid bilayer of cell membranes (Hayakawa et al., 1984).

Other types of enzymes, such as chitinases (QUIT), can also be produced by plants, animals, bacteria and fungi. They catalyze the hydrolysis of QUITin, a polysaccharide present in the cell wall of fungi, green algae and exoskeleton of crustaceans and insects. Thus, enzymatic lysis of the cell wall of phytopathogenic fungi through the action of extracellular chitinases becomes part of a biocontrol mechanism (Zarei et al., 2011).

Among the elicitors, medicinal plant such as *Lippia sidoides* Cham., have antimicrobial properties and can be studied for their elicitor potential (Da Silva et al., 2013). Both antioxidative enzymes and chitinases, besides being produced by plants, can be synthesized by a wide variety of endophytic fungi, such as *Beauveria*, *Lecanicillium*, *Metarhizium*, *Trichoderma* (ST Leger and Wang, 2010; Sandhu et al., 2017), *Nigrospora* and *Fusarium* exerting multiple effects on host plants (Yang et al., 2016).

Extracts of *L. sidoides* and their endophytic fungi may be used as a commercial product to reduce pest-related diseases. In this way, to identify the presence of enzymes that help in pest control, as well as the potential of phytoalexins induction is of extreme importance for the reduction of pesticides applied in organic plantations, mainly. If the extracts have this ability aforementioned, they probably can help crops of agricultural interest in their defense mechanisms against phytopathogens. Phytoalexins are considered important for plant resistance against pathogens. Plants are constantly attacked by many potential pathogens and respond by the activation of defense genes, the formation of ROS and the production of antimicrobial compounds. In this work, fresh leaves of *L. sidoides* and six endophytic fungi isolated from this plant were analyzed for the phytoalexin induction function, as well as the presence of enzymatic activity of SOD, APX, POX, CAT and QUIT in all extracts.

MATERIALS AND METHODS

Plant material, steam distillation and sample preparation

L. sidoides originating from Ceará was collected in Gurupi (11°44'48" latitude S, 49°02'55" longitude W), Tocantins, Brazil. Taxonomic identification was confirmed by experts at the herbarium (Federal University of São João Del Rei, Brazil), where samples were deposited with reference number 8303. As described by Guimarães et al. (2008), *L. sidoides* essential oil was extracted from the leaves by steam distillation method in a Clevenger-modified apparatus, as described by Guimarães et al. (2008) and stored at 4°C until further analysis. For the elicitor potential tests, essential oil concentrations of 0.625 to 7.5 mg mL^{-1} were prepared by adding Tween 80 (0.03%) and sterilized distilled water for solubilization.

Preparation of endophytic fungi extracts

The fungi *Cladosporium* sp., *Verticilium* sp., LS-6 (unidentified), *Colletotrichum* sp., *Fusarium* sp. 1, *Fusarium* sp. 2 and LS-14 (unidentified) were isolated from *L. sidoides* leaves. The mycelia of the endophytes were inoculated in 200 mL of pre-fermentative medium (Jackson et al., 1993). The cultures were incubated initially for 48 h under constant stirring (120 rpm) at 30 ± 1°C. Then, the mycelial masses were aseptically collected by vacuum filtration and reinoculated in 400 mL of the Czapek fermentation medium (Alviano et al., 1992). After 6 days of incubation under constant stirring (120 rpm) at 30 ± 1°C, the culture fluids were separated from the mycelia masses by vacuum filtration and the extract was obtained according to the methodology of Dhankhar et al. (2012). The extractions were carried out using ethyl acetate in order to

obtain the highest number of active antimicrobial substances. To obtain the positive control, the commercial systemic resistance activator, Biozyme® at 1.25 mg mL^{-1}, was used and as the negative control, distilled water + Tween 80 was used.

Test for phytoalexin induction in soybean cotyledons

Soybean seeds (*G. max*), cultivar Monsoy 8644-IPRO (Intacta®), were disinfected for 10 min in 1% sodium hypochlorite and washed in distilled water. Subsequently, they were sown in two trays containing autoclaved sand (121°C and 1 atm for 20 min). The trays were left in a greenhouse for 10 days and the cotyledons were then highlighted for the trials (Stangarlin et al., 2010).

The cotyledons were placed in 120 mm diameter Petri dishes, where each plate contained 3 cotyledons and 2 sheets of sterile filter paper and were moistened with sterile distilled water. Each cotyledon was cut into small fragments, which were treated with 100 µL of the samples to be tested. Each endophytic fungus extract was tested, being 16.3; 15.3; 82.3; 13.0; 13.4 and 14.0 mg mL^{-1} for the fungi *Cladosporum* sp., *Verticilium* sp., LS-6, *Colletotrichum* sp., *Fusarium* sp. 1, *Fusarium* sp. 2 and LS-14, respectively. In addition, five concentrations of the essential oil of *L. sidoides* were tested. The plates were incubated at 25 ± 1°C in the dark for 20 h. Then, the cotyledons were weighed and placed in Erlenmeyers containing 10 mL of sterile distilled water, which were left under orbital shaking (150 rpm) for 1 h for extraction of the pigments (Meinerz et al., 2008; Stangarlin et al., 2010). Finally, the cotyledons were removed from the Erlenmeyers and the absorbance of the supernatant was read in a spectrophotometer (BioSpectro model SP-220) at 285 nm (Meinerz et al., 2008). Sterilized distilled water together with Tween 80 were used as a negative control and the commercial resistance activator Acorda® (JUMA AGRO) was taken as positive control (Stangarlin et al., 2010).

Phytoalexin induction in sorghum mesocotyls

Sorghum (*S. bicolor*) seeds, Buster (Atlantic Seeds®), were placed in 1% sodium hypochlorite for 15 min and then washed in sterile distilled water. Then, the seeds were wrapped in moistened germination paper sheets and the leaves placed in upright bins, incubated in the dark at 28 ± 1°C within 4 days for germination to occur (Stangarlin et al., 2010). After this period, the seedlings formed were first exposed to light for 4 h so that the mesocotyl elongation would stop. In the sequence, the seedlings were sprayed with 2 mL of the samples to be tested (the same as the previous item). Sterilized distilled water + Tween 80 were used as the negative control, while the commercial resistance activator Biozyme® (Arysta Life Science) was the positive control. The seedlings were kept under fluorescent light at 25 ± 1°C for 60 h. Subsequently, the mesocotyls were removed, excised, weighed and placed in microcentrifuge tubes containing 1.4 mL of acidified 80% methanol (0.1% HCL; v/v). These were kept cooled to 4 ± 1°C per 96 h for the extraction of the pigments (Bonaldo, 2005). The absorbance was read at 480 nm (BioSpectro model SP-220) (Nicholson et al., 1988; Stangarlin et al., 2010).

Enzymatic activity

Determination of the enzymatic activity was performed both in the fresh leaves of *L. sidoides* and in the supernatant of the fermented medium of the six endophytic fungi associated with this plant which showed better results in the antifungal effect of *Curvularia lunata* (Wakker) (data not shown). Samples of *L. sidoides* were prepared by weighing 200 mg of the fresh leaves macerated in liquid nitrogen with 20% polyvinylpolyrolrolidone (PVPP). Upon formation of the powder, 1.5 mL of extraction buffer (100 mM potassium phosphate buffer, pH 7.0 added to 1 mM EDTA and 1 mM ascorbate to the volume of buffer used) was added. It was macerated for an additional 3 min and centrifuged at 14,000 *g* for 25 min at 4 ± 1°C. The supernatant was collected (protein extract) and stored at -20°C for further analysis. On the other hand, samples of the fungal extracts, that is, the liquid fermented by the fungi, were separated from the mycelia and conditioned directly in tubes for microcentrifuge at -20°C until the analyses were performed.

Superoxide dismutase (SOD; EC 1.15.1.1)

SOD activity was determined by inhibition of blue formazan production by nitroblue tetrazolium (NBT) photoreduction. The SOD was measured by adding 0.1 mL of protein extract in test tubes: 0.1 mL of 50 mM potassium phosphate buffer, pH 7.8; 0.02 mL of 0.1 mM EDTA; 0.4 mL of 70 mM L-methionine and 0.2 ml of 1 mM NBT. The reaction was started by adding 2 mM riboflavin and rapidly transferring the tubes, without light protection, to a 30 W lamp-lit chamber (30 µmol of photons $m^{-2}s^{-1}$) for 5 min. The absorbance was measured at 540 nm (BioSpectro model SP-220) (Giannopolotis and Ries, 1977). A unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT reduction and activity was expressed in unit per gram per extract per minute (U g^{-1} E^{-1} min^{-1}), according to Beauchamp and Fridovich (1971).

Peroxidase phenols (POX; EC 1.11.1.7)

POX activity, according to the method proposed by Amako et al. (1994) was measured by adding 0.05 mL of the protein extract to a test tube; 2.9 mL of solution with 0.5 mL of 0.2 M guaiacol and 0.5 mL of 0.38 M hydrogen peroxide and 2 mL of 0.02 M sodium acetate buffer; pH 5.0. The readings were performed at 470 nm (BioSpectro model SP-220), intercalating 15 s for 3 min (12 readings) for each reaction. To reset the device, a blank was made by adding distilled water to the sample. The POX activity was expressed in micromole of hydrogen peroxide per gram per extract per minute (µmol H_2O_2 g^{-1} E^{-1} min^{-1}).

Catalase (CAT; EC 1.11.1.6)

To determine CAT activity, 0.05 mL of protein extract was added; 2.95 mL of 50 mM potassium phosphate buffer, pH 7.8 with 20 mM hydrogen peroxide was added. The readings were recorded according to the decay of the absorbances. The activity was measured at 240 nm (BioSpectro model SP-220) for 300 s with readings performed every 30 s and calculated based on the molar extinction coefficient of 35 M^{-1} cm^{-1} (240 nm) and expressed as micromol hydrogen peroxide per gram per extract per minute (µmol H_2O_2 g^{-1} E^{-1} min^{-1}) (Havir and Mchale, 1987).

Ascorbate peroxidase (APX; EC 1.11.1.11)

APX activity enzyme was measured by mixing 0.1 mL of the protein extract, 2.7 mL of 0.5 mM ascorbate (ASA) buffer and 0.2 mL of 30 mM hydrogen peroxide. The absorbance was measured at 290 nm (BioSpectro model SP-220) at 25°C by the degradation of hydrogen peroxide. APX activity was expressed as micromol of ascorbate per gram per extract per minute (µmol ASA g^{-1} E^{-1} min^{-1}) (Asada and Takahashi, 1987).

Chitinase (QUIT; EC 3.2.1.14)

To determine QUIT activity, the release of soluble fragments of

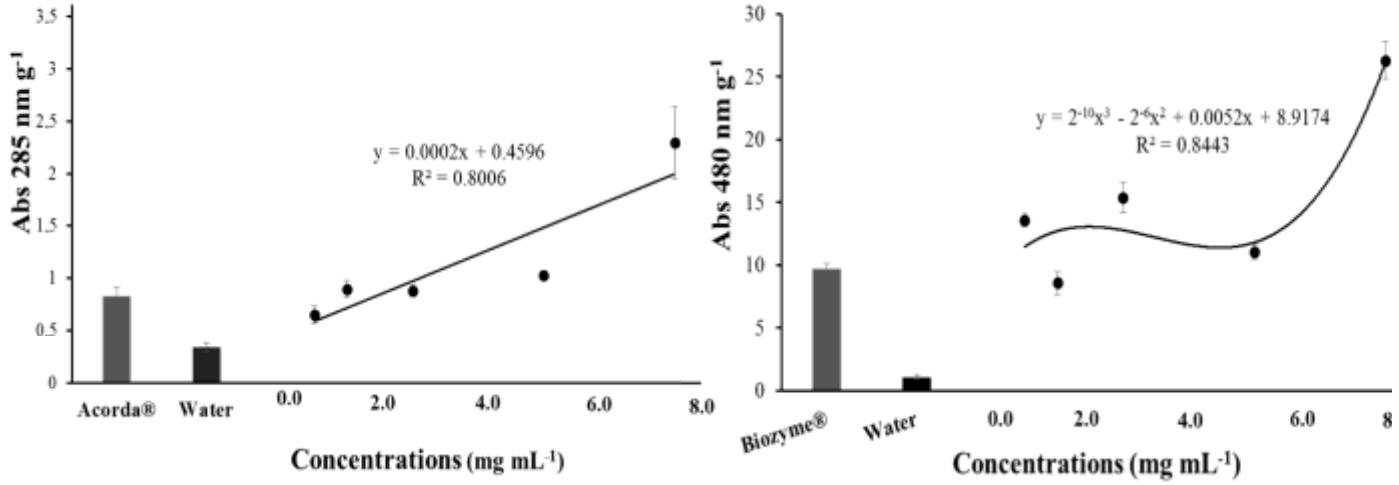

Figure 1. Accumulation of glyceollin in soybean cotyledons (A) and 3-deoxythianocyanidine (B) as a function of the application of different concentrations of *L. sidoides* essential oil.

"CM-QUITin-RBV®" (Sigma-Aldrich) was observed by means of carboxymethylated QUITin labeled with bright violet remazol. For the reaction, 0.2 mL of the protein extract, 0.6 mL of 0.1 M sodium acetate buffer pH 5.0 and 0.2 mL of "CM-QUITin-RBV®" was added to 2.0 mg mL^{-1}. Subsequently, it was incubated at 40°C for 20 min. The reaction was quenched with the addition of 0.2 mL of 1 M HCl, cooled on ice and centrifuged at 10,000 *g* for 5 min. The absorbance was measured at 550 nm (BioSpectro model SP-220). The activity of the QUIT enzyme was expressed in units of absorbance min^{-1} (U min^{-1}).

Statistical analysis

The data were analyzed by ANOVA and, when significant, the averages were compared by the Tukey test for the qualitative factor and regression analysis for the quantitative factor, 5% error probability, with the aid of statistical analysis software ASSISTAT and SIGMAPLOT 12.0. The graph in the heatmap was performed on the software Gitools 2.3.1.

RESULTS

The assays with *L. sidoides* essential oil had a dose-dependent effect, with adjustment of the 1st-degree equation and R^2 of 0.8006 for the production of phytoalexin glyceollin in soybean cotyledons (Figure 1A). All the concentrations tested (0.625, 1.25, 2.5, 5.0 and 7.5 mg mL^{-1}) provided a synthesis of the phytoalexin with the highest induction value at the concentration of 7.5 mg mL^{-1}. In addition, this concentration induced 66.48% more phytoalexin than the Acorda® positive control (Figure 1A).

Phytoalexin 3-deoxythyanocyanidine induction was observed in sorghum mesocotyls (Figure 1B) production at all tested concentrations (0.625, 1.25, 2.5, 5.0 and 7.5 mg mL^{-1}). In the regression analysis, it was possible to obtain the R^2 value of 0.89 with a 3rd-degree equation having no dose-dependent effect (Figure 1B). The maximum value obtained was obtained in the concentration of 2.5 mg mL^{-1}, with result of 28.25% higher than the Biozyme® positive control.

Among endophytic fungi extracts associated with *L. sidoide*s tested in this work, the one that presented greater result was the *Fusarium* sp. 1. This fungus induced 25.29% more phytoalexins than the positive control and 29.79% more than the *Verticillium* sp. fungus, which showed the lowest value (Figure 2A).

The endophytic fungus, *Verticillium* sp. was the one that showed the best result regarding the production of 3-deoxythianocyanidine, and it induced 93.83% more phytoalexins than the fungus *Fusarium* sp. 2, which showed lower result, and 66.64% more than the negative control (Figure 2B).

The results in Figure 3A show that there was activity of the SOD enzyme, where the highest value, 130 U g^{-1} E^{-1} min^{-1}, was presented by the fungus LS-14. This value is higher than that of the *L. sidoides* plant, 64 U g^{-1} E^{-1} min^{-1}. Regarding the activity of the QUIT enzyme, the highest value of 0.214 U min^{-1} was obtained for the *Fusarium* 1 fungus. It is also noted that, both the *L. sidoides* plant and the fungus *Verticillium* sp. did not synthesize the enzyme chitinase since no activity was detected (Figure 3B).

For APX activity, the highest value was 3821 µmol ASA g^{-1} E^{-1} min^{-1} for *L. sidoides* (Figure 4A). For the activity of CAT, the featured was also for *L. sidoides* with 2429 µmol H_2O_2 g^{-1} E^{-1} min^{-1} of catalytic activity determination, however, no significant value of activity was observed for the fungus *Colletotrichum* sp. (Figure 4B). In relation to the POX activity, it can be observed that there was a contrast since the lowest value was for *L. sidoides* (2 µmol H_2O_2 g^{-1} E^{-1} min^{-1}) and higher value for the fungus *Fusarium* sp. 2 was 87 µmol H_2O_2 g^{-1} E^{-1} min^{-1} (Figure 4C).

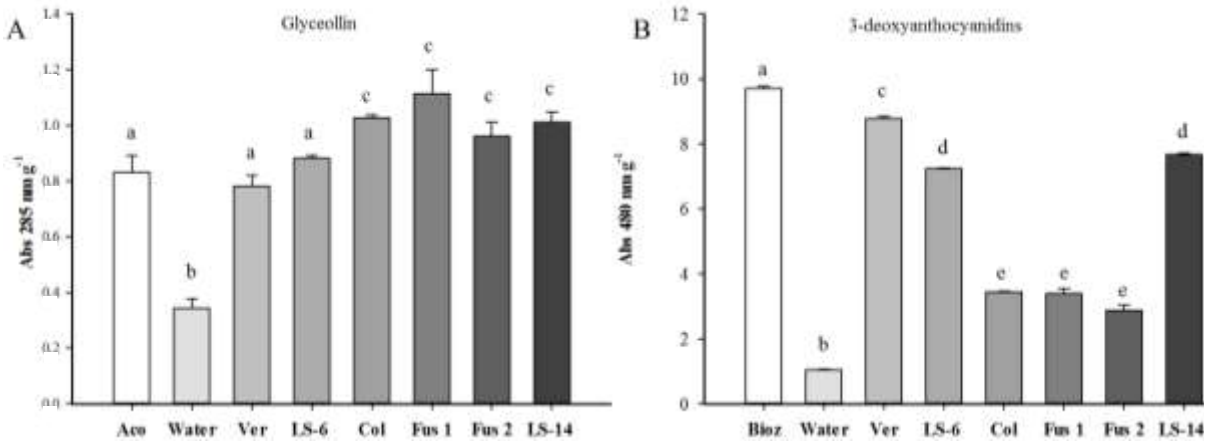

Figure 2. Production of glyceollin in soybean cotyledons (A) and 3-deoxythyanocyanines in sorghum mesocotyls (B) subjected to treatment with endophytic fungi extract. Means accompanied by the same letter do not differ by the Tukey test at 5% probability.

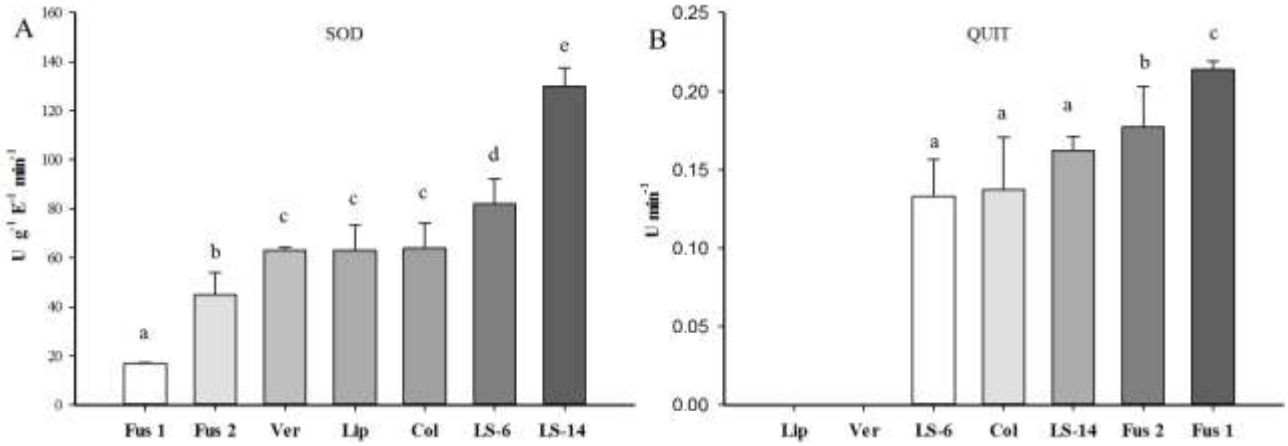

Figure 3. Enzymatic activity of superoxide dismutase SOD (A) and chitinase QUIT (B) in the fermentative supernatant of different cultures of endophytic fungi associated with *L. sidoides*. Means with the same letter do not differ by the Tukey test at 5% probability.

Figure 5 shows the data of the enzymatic activity in heatmap graph format. It is a way of condensing information, where the columns correspond to the enzymes and the lines correspond to the extracts of both the plant and the six endophytic fungi studied. The colored tiles indicate the intensity of the enzymatic activity. It is shown in Figure 5 that the highest enzymatic activity was obtained with respect to the APX enzyme for *L. sidoides* extract and the smallest enzymatic activities are represented by QUIT.

DISCUSSION

Among the defense activators of the tested plants, Acorda® induced the highest production of glyceollin in soy cotyledons, and it was used as a positive control (data not shown). For the sorghum tests, it was found that the Biozyme® activator generated better results for the induction of flavonoid 3-deoxythyanocyanidine (data not shown).

Due to this bioactive and beneficial capacity for plants and other organisms, phytoalexin synthesis tests induced by endophytic fungal extracts were similarly performed. Previously, these extracts were screened against the phytopathogenic fungus *C. lunata*. The six extracts that inhibited *in vitro* mycelial growth of phytopathogen (Ferreira et al., 2017) were separated for the phytoalexin production tests.

Therefore, all the endophytic fungi extracts associated with *L. sidoide*s tested in this work demonstrated similar or greater induction of glyceollin than the positive control.

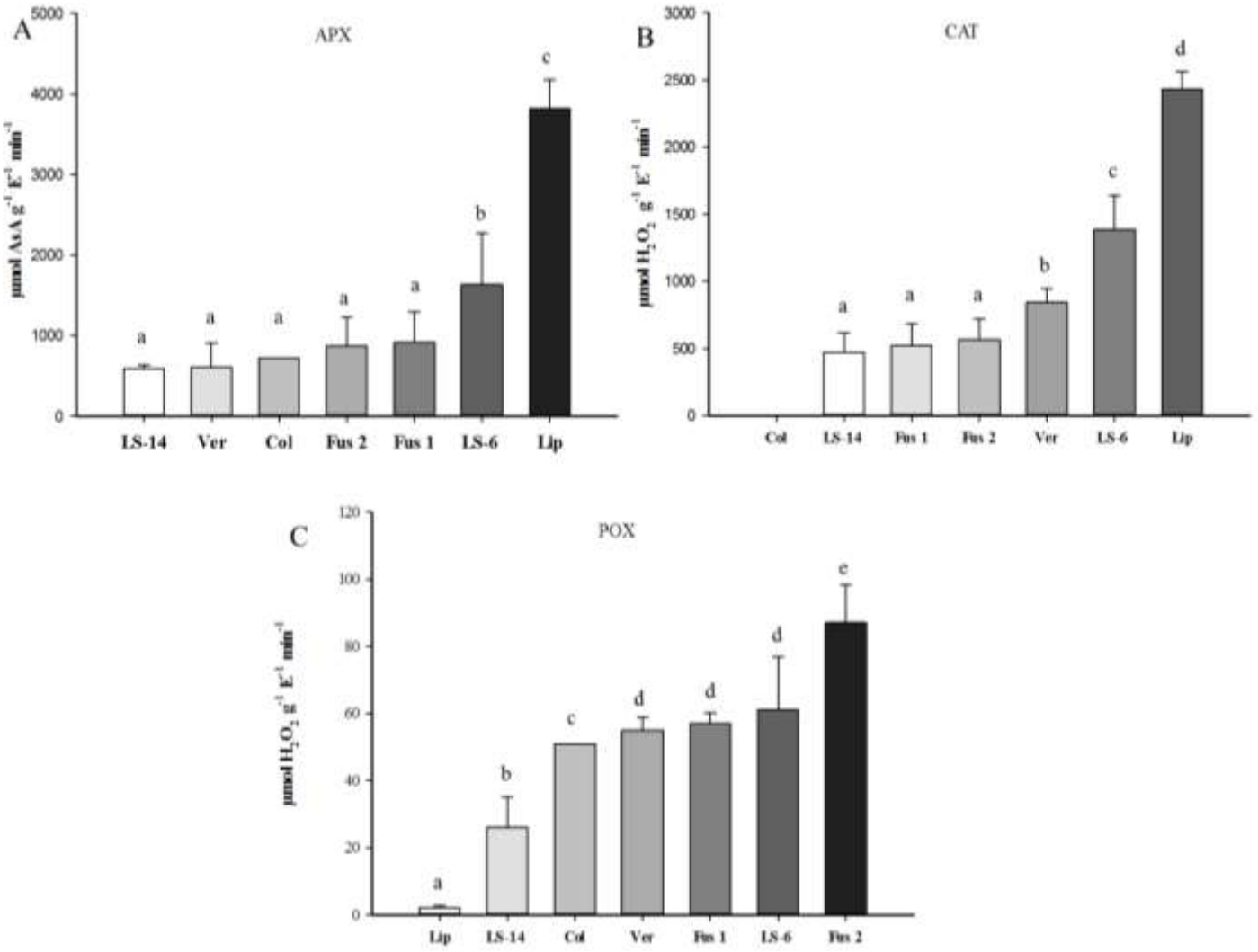

Figure 4. Enzymatic activity of ascorbate peroxidase APX (A), catalase CAT (B) and phenol peroxidase POX (C) in the fermentative supernatant of different cultures of endophytic fungi and *L. sidoides*. Means with the same letter do not differ among themselves by the Tukey test at 5% probability.

Bonaldo et al. (2007) observed that the essential oil of *Eucalyptus citriodora* at the tested concentrations of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} did not promote the synthesis of phytoalexins in sorghum in contrast to the present work. However, the crude extract of the same plant induced accumulation of 3-deoxythianocyanidine. They believe that the negative result is because the solutions are quite diluted.

Other studies with leaf extracts of *Ocimum gratissimum* L. (alfavaca) induced the production of 3-deoxythianocyanidine when the 40% w/v concentration was used. Also, the same extract induced 6-fold more glyceollin synthesis than the control (distilled water) at 25% w/v concentration. In this research, the production of the two phytoalexins increased initially and then, with increase of the extract concentration, there was a decrease in the production. The authors indicated that phytoalexins were inactivated, possibly by irreversible binding to cell debris, which cannot be removed or detected (Colpas et al., 2009). This may also have occurred with the production of glyceollin from the concentrations of 5.0 and 7.5 mg mL^{-1} of *L. sidoides* essential oil in the present study.

Glyceollin, phytoalexin derived from soybeans, exists as three mixed isomers. It is released at high concentrations during plant growth in response to a number of stress factors, such as injury, freezing, exposure to ultraviolet light, chemicals and exposure to microorganisms (Feng et al., 2007). Several studies have shown that its biological activities included antitumor, antiestrogenic, antibacterial and antifungal effects (Liu et al., 2014). Therefore, the analysis of its effects and its production are necessary to obtain an alternative type of control with the use of essential oils.

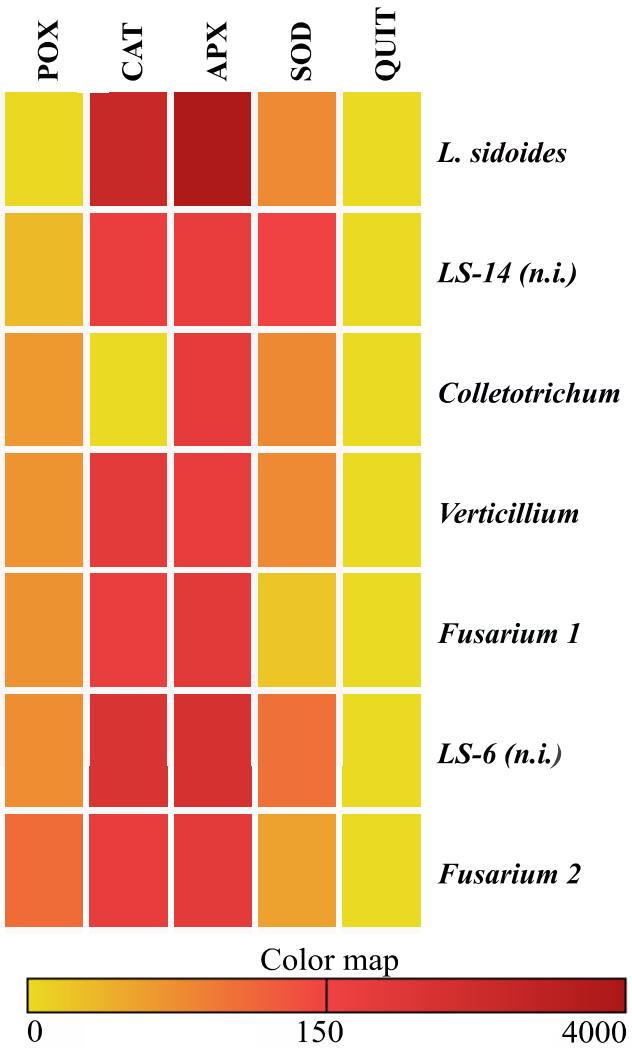

Figure 5. Heat map of the enzymatic activity values obtained for the enzymes POX, CAT, APX, SOD and QUIT in the analysis of the extracts of *Lippia sidoides* and the extracts of the endophytic fungi, *Verticillium* sp., *Colletotrichum* sp., *Fusarium* 1, *Fusarium* 2, LS-6 and LS-14.

No similar results were found in relation to endophytic fungi extracts. However, Arruda et al. (2012) used an aqueous extract of mushrooms such as *Agaricus blazei* at 10% concentration and observed the induction of glyceollins. The accumulation from the concentration of 1 and 2% for extracts of *Lentinula edodes* was also evidenced; the induction was observed for the extracts of *Pycnoporus sanguineus*. Piccinin et al. (2000) used filtration of *L. edodes* basidiocarp autoclaved, filtered mycelial growth, filtered macerated mycelium and observed the induction of 3-deoxyanthiocyanins in mesocotyls of sorghum and glyceollins in soybean cotyledons.

A small production of phytoalexins was observed in the negative control in which distilled water was used (Figures 1A, B, 2A and B). This small synthesis may be due to the mechanical lesions in the cotyledons to perform the tests. As phytoalexins appear after infection of the pathogen, it may be suggested that endophytic fungi synthesized molecules, releasing them from their hyphae into the fermentative medium (Hammerschmidt, 1999), which could be washed away with the solvent, ethyl acetate. These substances, when in higher concentrations, make the perception of signals derived from the elicitor more efficient, causing alterations in the cellular metabolism, such as activation of G proteins, increase in the ion flow through the plasma membrane, activity of kinases and phosphatases, activation of metabolic routes, among them, the synthesis of phytoalexins (Mazaro et al., 2008).

Typically, 2 to 5% of O_2 is reduced univalent, a process in which a molecule receives only one electron, which will occupy one of the outer orbitals while the other remains unpaired. The consequence of this is the production of highly reactive intermediates, called reactive oxygen species (ROS), free radicals. Thus, the first reactive toxic species of oxygen, superoxide ($O_2^{\cdot -}$), is formed. This radical can be disrupted in hydrogen peroxide (H_2O_2) or even through catalytic action by the action of the enzyme SOD (Halliwell and Gutteridge, 1989).

Thus, by identifying the presence of enzymatic activity of SOD in the fermentative products of endophytic fungi associated with *L. sidoides*, it is possible to establish a real, mutual and synergistic relationship of these organisms. The plant favors the growth of these fungi in its interior as the comfortable habitat for the life cycle of these microorganisms, and also, the fungi also protect it by helping in the elimination of these ROS. Therefore, through the enzymatic activities performed in the present study, this was observed. All fungal samples contained enzyme activity.

Nunes et al. (2006), observed an increase in the mean values of the enzymatic activity of SOD when the combination of the phytohormones 6-benzylaminopurine acid (BAP) and naphthaleneacetic acid (ANA) was used, which led to the accumulation of ROS due to oxidative stress. There are also studies on endophytic fungi of wine grape, which were reinoculated to show the antioxidative activities. It includes the enzymatic activity of SOD for a possible application of endophytic fungi as metabolic regulators in winemaking (Yang et al., 2016). There are no reports on chitinase activity in *L. sidoides* leaves in the literature. Souza et al. (2001) found endochitinases produced by the fungus, *Colletotrichum gloeosporioides*. The *Fusarium* fungus has no history of chitinase production. Brzezinska and Jankiewicz (2012) reported on the use of chitinases against phytopathogenic *Fusarium culmorum* and *Fusarium solani*. It is important that an organism synthesizes chitinases since it has been demonstrated that the overexpression of chitinase genes in plants increases resistance to pathogens. This enzyme acts on the hydrolysis of QUIT in polymers, whose substances are part of the fungal cell walls (Van Loon et al., 2006). Therefore, it would be a great antimicrobial.

The activity of APX, CAT and POX was also performed for both the *L. sidoides* plant and the associated endophytic fungi. These tests were also done in order to detect the presence of these enzymes and to infer that endophytic fungi really help, protect and live in synergy with the host plant. The enzymes APX, CAT, POX and SOD are among the enzymes considered antioxidative. They can be found in virtually all cell sites and plants, and participate in the hydrogen peroxide detoxification system (Asada, 1992). Determination of the activity of these enzymes plays an important role in elucidating the main functions of the endophytic fungi inside their host plants. They contribute to the combined actions of the plant and fungus. In addition, ROS lead to the oxidation of biomolecules with consequent loss of their biological functions and/or homeostatic imbalance, causing damage to plant cells and tissues such as fungi (Halliwell and Whiteman, 2004). All fungal samples contained enzyme activity.

In the work of Pimenta et al. (2013), these enzymes were detected in the plant, *Lippia filifolia*, where they verified the effect of the inhibition of ethylene synthesis on oxidative stress. The expression of the genes encoding these enzymes is dependent on several types of abiotic and biotic stresses emphasizing the importance and complexity of understanding the mechanisms involved and their complexity. Both enzymes and antioxidative metabolites may be able to prevent the accumulation of ROS and oxidative stress (Saher et al., 2004), serving as the signal to prevent abnormalities (Nunes et al., 2015). Further study will be carried out to verify the antimicrobial activity of the phytoalexins extracted from the extracts. There is also a need to establish other methodologies for extracting phytoalexins in corn crop, for example. By means of real-time PCR analysis, synthesis of the POX, CAT, APX, SOD and QUIT enzymes can be quantitatively verified by the endophytic fungi and by the plant, as well as to verify the induction of the synthesis of these enzymes in cultures of agricultural interest before and after the application of these extracts. Research related to the induction of defense and/or resistance substances that are natural are important and necessary for use in agriculture. They can be cheaper, more efficient, biodegradable and do not cause harm to the environment.

Conclusions

The essential oil of *L. sidoides* and all extracts of the endophytic fungi associated with it can be considered elicitors of phytoalexins by the presence of molecules that indicate triggering of the biosynthesis of the same ones. In addition, the presence of enzymes related to antioxidative activity such as SOD, APX, POX and CAT and related to the hydrolysis of QUIT in polymers, such as in both leaves of the plant and the fermented liquid of the endophytic fungi, were verified and proved. In this way, it has important and potential commercial products for activating plant defense and promoting its resistance in cultivars of agricultural interest, but mainly organic farming.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

SOD, Superoxide dismutase; **QUIT,** chitinase; **APX,**

ascorbate peroxidase; **POX,** phenol peroxidase; **CAT,** catalase; **Ver,** *Verticillium* sp.; **Col,** *Colletotrichum* sp.; **Fus 1,** *Fusarium* sp. 1; **Fus 2,** *Fusarium* sp. 2; **Lip,** *Lippia sidoides*; **LS-6,** code of the unidentified fungus; **LS-14,** code of the unidentified fungus; **ROS,** reactive oxygen species.

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